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Quantitative disease resistance to the bacterial pathogen *Xanthomonas campestris* involves an Arabidopsis immune receptor pair and a gene of unknown function

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SUMMARY

Although quantitative disease resistance (QDR) is a durable and broad-spectrum form of resistance in plants, the identification of the genes underlying QDR is still in its infancy. *RKS1* (*Resistance related KinaSe1*) has been reported recently to confer QDR in *Arabidopsis thaliana* to most but not all races of the bacterial pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*). We therefore explored the genetic bases of QDR in *A. thaliana* to diverse races of *X. campestris* (*Xc*). A nested genome-wide association mapping approach was used to finely map the genomic regions associated with QDR to *Xcc12824* (race 2) and *XccCFBP6943* (race 6). To identify the gene(s) implicated in QDR, insertional mutants (T-DNA) were selected for the candidate genes and phenotyped in response to *Xc*. We identified two major QTLs that confer resistance specifically to *Xcc12824* and *XccCFBP6943*. Although QDR to *Xcc12824* is conferred by *At5g22540* encoding for a protein of unknown function, QDR to *XccCFBP6943* involves the well-known immune receptor pair RRS1/RPS4. In addition to *RKS1*, this study reveals that three genes are involved in resistance to *Xc* with strikingly different ranges of specificity, suggesting that QDR to *Xc* involves a complex network integrating multiple response pathways triggered by distinct pathogen molecular determinants.

Keywords: *Arabidopsis thaliana*, GWA mapping, mutant analysis, quantitative disease resistance, *RRS1*/*RPS4*, *Xanthomonas campestris*.

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INTRODUCTION

A fundamental constraint on agricultural productivity is the responsiveness of plants to biotic stress in their natural environment. Plants are under continuous stress caused by different bioaggressors (e.g. bacteria, fungi, viruses, oomycetes, nematodes and insects) that compromises plant performance and survival. Consequently, plants have evolved complex resistance mechanisms that are constitutively expressed or induced after pathogen attack (Glazebrook, 2005; Panstruga *et al*., 2009). Defence traits are not cost free (Bergelson *et al*., 1996; Vila-Aiub *et al*., 2011), most probably because metabolite allocation to resistance may negatively impact growth and yield components (Strauss *et al*., 2002). These physiological constraints, together with the co-existence between plants and natural pests, have driven the evolution of the plant innate immune system as a dynamic and complex network interconnecting different defensive layers in various cellular components (Chisholm *et al*., 2006; Panstruga *et al*., 2009; Schulze-Lefert and Panstruga, 2011).

Plants can detect conserved molecular signatures of the pathogen (pathogen-associated molecular patterns, or PAMPs) by host cell surface pattern recognition receptors, which activate pattern-triggered immunity (PTI). Successful pathogens deliver effector proteins into the host that suppress these defences (effector-triggered susceptibility, ETS). To counter ETS, plants possess a second layer of immune receptors [encoded by resistance (*R*) genes] that detect the presence of effectors, leading to effector-triggered immunity (ETI) (Jones and Dangl, 2006). Dominant *R*-gene-mediated ETI is considered to be the most efficient form of resistance in plants. Thus, our current knowledge of plant disease resistance mechanisms is largely derived from the characterization of *R* genes mediating the strain-specific recognition of biotrophic or hemibiotrophic pathogens. This has been a powerful approach to understand fundamental processes in plant–pathogen recognition. *R* genes that have been most extensively characterized express a highly effective local resistance response that is often associated with hypersensitive cell death (the hypersensitive

response, or HR). Consequently, this creates a strong evolutionary pressure for the selection of virulent pathogen strains that can bypass recognition (McDonald and Linde, 2002).

Because ETI fails to provide durable and broad-spectrum resistance in an agricultural context in most cases, increasing attention has been devoted recently to quantitative disease resistance (QDR; Fukuoka *et al*., 2015; Roux *et al*., 2014a). In addition, QDR is much more prevalent than specific resistance in crops and natural plant populations (Young, 1996; Zuo *et al*., 2015). QDR is a form of resistance which leads to a reduction in disease, rather than an absence of disease (Poland *et al*., 2009), and is typically polygenic (Roux *et al*., 2014a). Although numerous disease resistance quantitative trait loci (QTLs) have been identified in plants, the genes and associated mechanisms underlying QDR remain largely unknown. The recent cloning of a limited number of QDR genes underlying QTLs identified by traditional linkage mapping approaches has suggested that few QDR genes correspond to nucleotide-binding leucine-rich repeat (NB-LRR) genes (Broglie *et al*., 2011; Fukuoka *et al*., 2014; Staal *et al*., 2006). On the contrary, a broad range of molecular functions is represented by the different QDR genes identified so far (Balint-Kurti and Holland, 2015; Roux *et al*., 2014a). For instance, although the kinase-START WKS1 confers resistance to wheat stripe rust (Fu *et al*., 2009), the putative ABC transporter LR34 confers resistance to multiple fungal pathogens in wheat (Krattinger *et al*., 2009). In addition, the serine hydroxymethyltransferase RGH4 confers cyst nematode resistance in soybean (Liu *et al*., 2012). These molecular functions have not been associated previously with plant disease resistance, suggesting that the molecular mechanisms underlying QDR may be more diverse than anticipated (Roux *et al*., 2014a).

Recently, we have reported the identification, map-based cloning and functional validation of *RKS1* (*Resistance related KinaSe1*) conferring QDR in the model plant *Arabidopsis thaliana* to the bacterial vascular species *Xanthomonas campestris* (*Xc*), which is responsible for black rot, an important disease of crucifers worldwide (Huard-Chauveau *et al*., 2013). *RKS1* encodes an atypical kinase lacking some critical domains in the kinase catalytic core that are essential for catalysis (Roux *et al*., 2014b). Interestingly, atypical kinases (or pseudokinases) have been described as important regulators of signalling networks. Furthermore, in addition to the presence of polymorphic populations across the native range of *A. thaliana*, a signature of balancing selection acting on *RKS1* with the active maintenance of two highly divergent haplotypes on an evolutionary time scale highlights that QDR to *Xc* may be maintained as a long-lived polymorphism at the species level (Huard-Chauveau *et al*., 2013). Finally, we demonstrated that *RKS1* confers a broad-spectrum resistance to *Xc*. *RKS1*-dependent immunity is effective not only against the strain *Xcc568* of *Xanthomonas campestris* pv. *campestris* (*Xcc*, race 3), but also against strains in four additional races of *Xcc* (races 1, 5, 7 and 9). *RKS1* has also been found to confer resistance to additional pathovars of *Xc: raphani* (*Xcr*), *armoriaceae* and *incanae*.

Because *RKS1* does not confer resistance to all races of *Xcc*, in this study, we explored the *RKS1*-independent genetic bases of quantitative resistance in *A. thaliana* to diverse races of *Xcc*. Using a nested genome-wide association (GWA) mapping approach (Huard-Chauveau *et al*., 2013; Ogura and Busch, 2015) to finely map genomic regions associated with quantitative phenotypic traits, we identified two major QTLs that confer resistance specifically to *Xcc12824* (race 2) and *XccCFBP6943* (race 6). Through mutant analysis, we first identified the *At5g22540* gene conferring QDR to *Xcc12824* (race 2), which encodes a protein of unknown function. Interestingly, we identified the dual resistance gene system *RRS1/RPS4* (Narusaka *et al*., 2009a) as being involved in QDR to *XccCFBP6943* (race 6). Together with *RKS1*, these genes confer strikingly different recognition specificities to other races and pathovars of *Xc*, suggesting that QDR to *Xc* involves a complex network integrating multiple response pathways triggered by distinct pathogen molecular determinants.

RESULTS

Two major QTLs confer resistance specifically to *Xcc12824* (race 2) and *XccCFBP6943* (race 6)

Based on the phenotyping data obtained on 380 natural accessions of *A. thaliana*, we found substantial natural genetic variation for QDR to the strain *Xcc12824* [race 2; disease index at 10 days post-inoculation (dpi); accession effect $F = 4.32$, $P < 0.0001$; broad-sense heritability $H^2 = 0.81$], with a prevalence of resistant accessions (Fig. 1a). GWA mapping revealed a unique neat peak of association on the short arm of chromosome 5 (Fig. 1b), with SNP-5-7481857 being the most associated single-nucleotide polymorphism (SNP) [$P = 5.29 \times 10^{-34}$; minor allele relative frequency (MARF) = 0.478] and located in the gene *At5g22540*. To identify other putative association peaks, we adopted an approach of nested GWA mapping (Huard-Chauveau *et al*., 2013) by first splitting the set of 380 accessions by the C/A polymorphism at this top SNP. Although substantial genetic variation of the disease index was detected within the more susceptible (S) SNP-5-7481857-C allelic group (Fig. 1a), we found no obvious association peak (Fig. 1c). In contrast, although little genetic variation was found in the more resistant (R) SNP-5-7481857-A allelic group (Fig. 1a), we found a unique neat peak of association located 6 kb upstream from the first peak of association (Fig. 1d), with SNP-5-7475828 being the most associated SNP ($P = 2.74 \times 10^{-23}$; MARF = 0.083) and located in the gene *At5g22510*. Nested GWA mapping thus suggests a second susceptible allele, SNP-5-7475828-A, segregating within the R allelic group SNP-5-7481857-A. The three allelic groups (SNP-5-7481857-C, SNP-5-7481857-A/SNP-5-7475828-A,

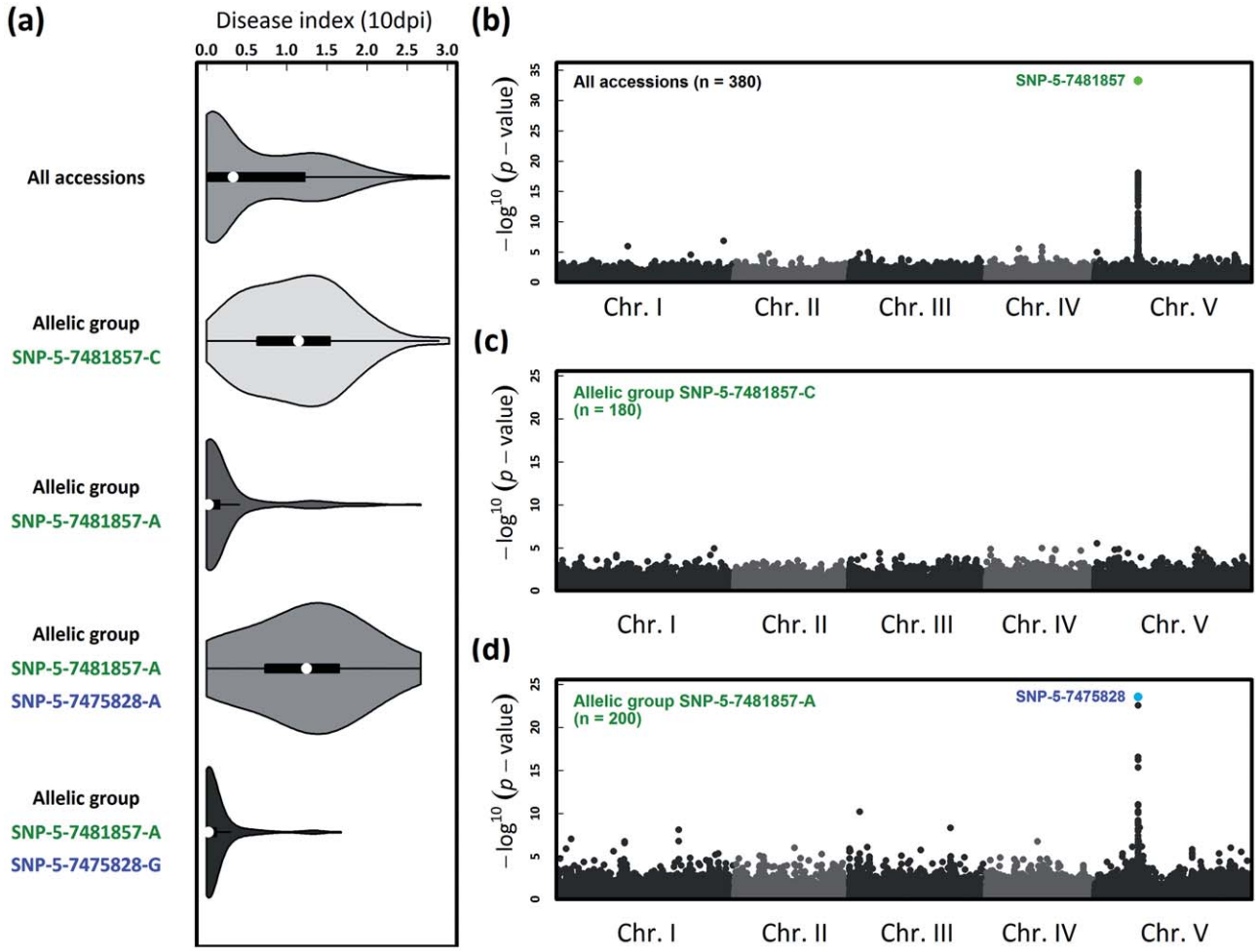

Fig. 1 The genetics of quantitative disease resistance to the strain *Xcc12824* identified by nested genome-wide association (GWA) mapping. (a) Violin plots (i.e. box-and-whisker plot overlaid with a kernel density plot) of the phenotypic variation of our disease index. Whole-genome scan of 214 051 single-nucleotide polymorphisms (SNPs) for association with disease index at 10 days post-inoculation (dpi) across (b) 380 accessions, (c) within the allelic group SNP-5-7481857-C and (d) within the allelic group SNP-5-7481857-A.

SNP-5-7481857-A/SNP-5-7475828-G) explained 51.1% of the natural genetic variation.

Based on the phenotyping data obtained on 171 natural accessions of *A. thaliana*, extensive natural genetic variation was also found for QDR to the strain *XccCFBP6943* (race 6; disease index at 10 dpi; accession effect $F = 5.01$, $P < 0.0001$; $H^2 = 0.80$; Fig. 2a). In contrast with the strain *Xcc12824*, most natural accessions were susceptible to the strain *XccCFBP6943* (Fig. 2a). GWA mapping revealed a unique peak of association on the long arm of chromosome 5 (Fig. 2b), with SNP-5-18325565 being the most associated SNP ($P = 1.41 \times 10^{-10}$; MARF = 0.246) and located in the gene *At5g45250*. After splitting the set of 171 accessions by the G/A polymorphism at this top SNP, extensive genetic variation for QDR was still observed within both allelic groups (Fig. 2a). No obvious association peak was found within the more resistant SNP-5-18325565-G allelic group (Fig. 2c). In the more susceptible SNP-5-18325565-A allelic group, we identified several peaks that were weakly associated with QDR (i.e. not significant at the stringent Bonferroni threshold, i.e. $P = 2.30 \times 10^{-8}$), including one association peak located in the 5' region of the gene *At5g45250* (SNP-5-18320658; $P = 1.12 \times 10^{-6}$; MARF = 0.300). Nested GWA mapping thus suggests an allelic series in the vicinity of *At5g45250*. The three allelic groups (SNP-5-18325565-G, SNP-5-18325565-A/SNP-5-18320658-A, SNP-5-18325565-A/SNP-5-18320658-T) explained 30.3% of the natural genetic variation.

QDR to *Xcc12824* (race 2) is conferred by the *At5g22540* gene

On the basis of the results of GWA mapping performed on the natural variation of QDR to *Xcc12824*, two candidate genes were identified: *At5g22540* corresponding to SNP-5-7481857 being the

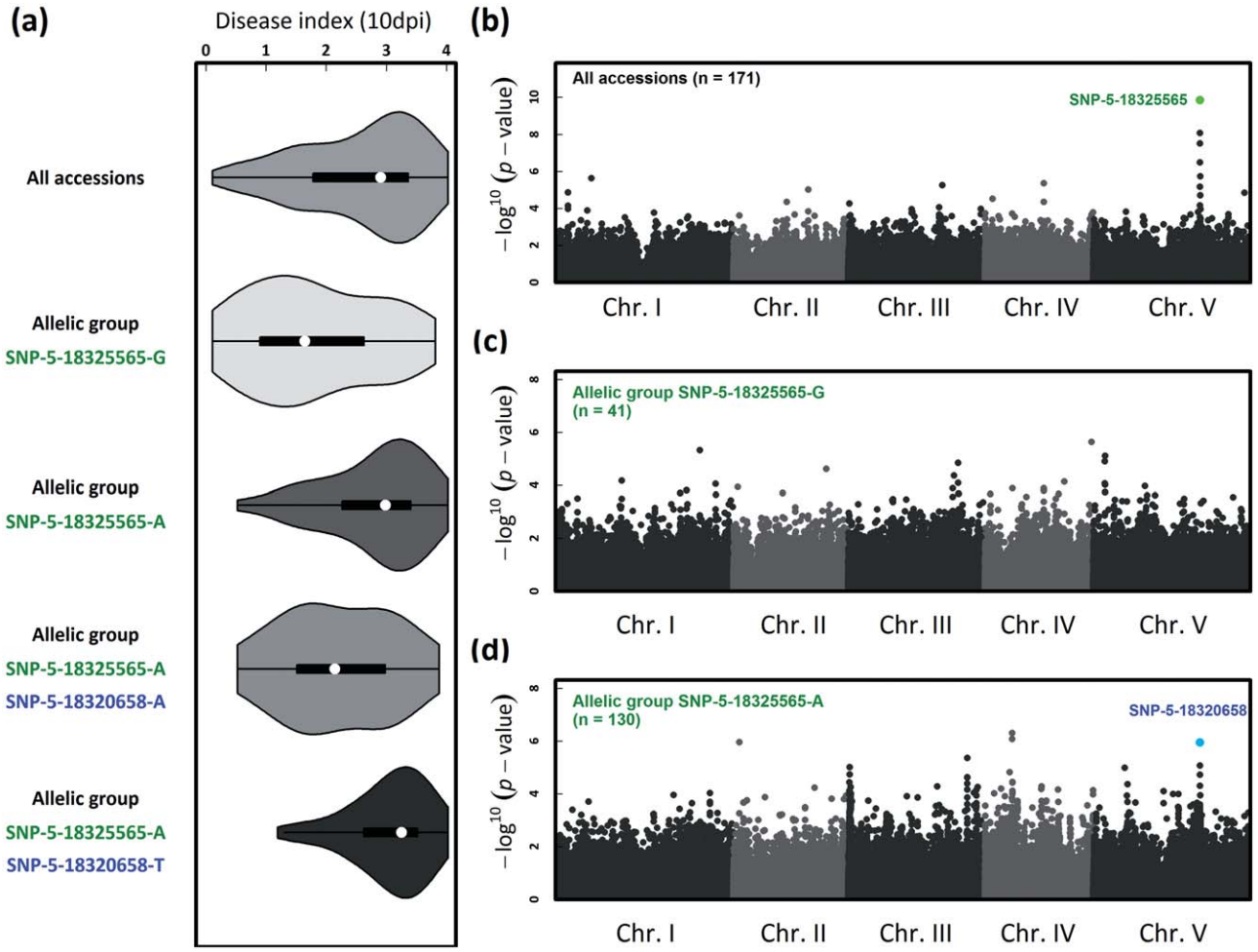

Fig. 2 The genetics of quantitative disease resistance to the strain *XccCFBP6943* identified by nested genome-wide association (GWA) mapping. (a) Violin plots (i.e. box-and-whisker plot overlaid with a kernel density plot) of the phenotypic variation of our disease index. Whole-genome scan of 214 051 single-nucleotide polymorphisms (SNPs) for association with disease index at 10 days post-inoculation (dpi) across (b) 171 accessions, (c) within the allelic group SNP-5-18325565-G and (d) within the allelic group SNP-5-18325565-A.

most associated SNP, and *At5g22510* corresponding to SNP-5-7475828 in the more resistant SNP-5-7481857-A allelic group. To identify the gene(s) implicated in resistance to *Xcc12824*, insertional mutants (T-DNA) in a *Xcc12824*-resistant Col-0 genetic background were selected for these two candidate genes, as well as for some other genes within the genomic region of interest when available (notably *At5g22500*, *At5g22530*). These mutants were inoculated with the strain *Xcc12824* and the disease index was evaluated at four time points after inoculation. Although the mutants *At5g22500-1* (*SALK N654537*), *At5g22500-2* (*SALK N667597*), *At5g22500-3* (*SALK N664670*), *At5g22510* (*SALK N670195*), *At5g22530-1* (*SALK N682427*) and *At5g22530-2* (*SALK N681954*) never showed disease symptoms, the mutant *At5g22540* (hereafter called the *mut540* mutant, *SALK-N667062*) exhibited a high level of susceptibility, which was, however, slightly lower than that of the susceptible accession Kas-1 (Table S1, see Supporting Information; Fig. 3a,b). These observations were confirmed by evaluation of *in planta* bacterial growth, with the gene *At5g22540* being involved in the resistance to bacterial colonization (Fig. 3c). In *mut540*, the T-DNA insertion located at position 1253 of the coding region leads to enhanced expression of the *At5g22540* gene (37-fold relative to Col-0 in healthy leaves; Fig. S1, see Supporting Information). *At5g22540* encodes a predicted protein of 440 amino acids, whose function is unknown. The expression of this gene was assessed during the interaction with *Xcc12824*, and showed a significant decrease during the first 6 h following bacterial inoculation (Fig. S1c). In addition, by testing different *Xcc* strains belonging to the nine races identified by Fargier *et al*. (2011) and diverse *Xc* strains belonging to *Xcr*, *At5g22540* appeared to be involved in resistance not only to race 2, but also, to a lesser extent, to races 1 and 4 at 7 dpi (Table 1). Interestingly, at later time points (10 dpi), resistance to race 3 (strain *Xcc568*) was also significantly affected in the mutant *mut540* (Table S2, see Supporting Information). As

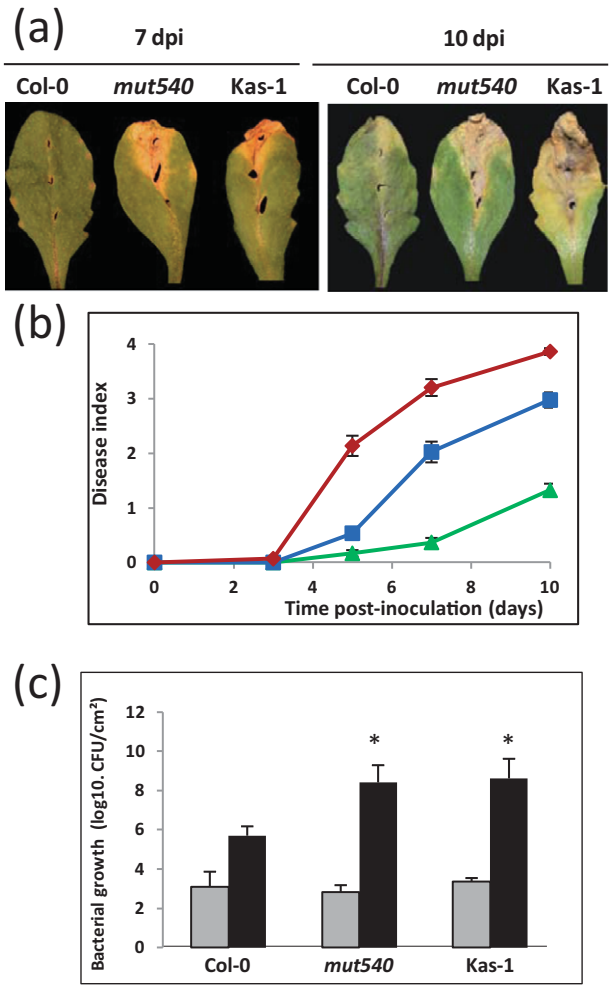

Fig. 3 The mutant *mut540* (affected in the *At5g22540* gene) is susceptible to *Xcc12824*. (a) Disease symptoms were observed at 7 and 10 days post-inoculation (dpi) on leaves of wild-type and *mut540* mutant (*SALK 113262C*) plants inoculated with *Xcc12824* (race 2 defined by Fargier *et al*., 2011 and Vicente *et al*., 2001). (b) Time course evaluation of disease index was performed in the *mut540* line (blue) relative to the susceptible accession Kas-1 (red) and the resistant accession Col-0 (green), after inoculation with *Xcc12824*. Means and standard errors were calculated from 8–15 plants (three independent experiments). (c) Bacterial growth measurement [colony-forming units (CFU)/cm^2 expressed on a $\log_{10}$ scale] in leaves of the *mut540* line relative to the wild-type accession Col-0. The susceptible accession Kas-1 was included as a positive control. Bacterial growth was measured 0 dpi (grey bars) and 7 dpi (black bars) with *Xcc12824*. Data were collected from two independent experiments; each time point corresponds to six independent measurements, each on three to five individual plants (four leaves/plant). *Statistically significant difference using Kruskal–Wallis test ($P < 0.05$).

demonstrated previously (Huard-Chauveau *et al*., 2013), the mutant *rks1-1*, used as a control in these experiments, was affected in resistance to races 1, 3, 5, 7 and 9 (and, to a much lesser extent, to race 8) and to strains belonging to *Xcr*, but not to races 2 and 4 (Table 1).

Table 1 Disease index (7 days post-inoculation) of the *mut540* mutant after inoculation with different strains belonging to *Xanthomonas campestris* pv. *campestris* (*Xcc*) races, as defined by Fargier *et al*. (2011) and Vicente *et al*. (2001), and to the pathovar *raphani* (*Xcr*).

Mutant accession	*Xcc1869* (race 1)*	*Xcc12824* (race 2)*	*Xcc568* (race 3)*	*Xcc147*† (race 4)*	*Xcc1712* (race 5)†	*Xcc6943* (race 6)*	*Xcc4953* (race 7)†	*Xcc1124* (race 8)*	*Xcc8004* (race 9)†	*Xcr756C*†	*Xcr5828*†	*Xcr7144*†
mut540	**1.39 ± 0.19^b**	**2.02 ± 0.19^b**	0.58 ± 0.19^a	**0.84 ± 0.17^b**	0.58 ± 0.18^a	1.58 ± 0.32^a	0.67 ± 0.22^a	0.17 ± 0.11^a	0.92 ± 0.22^a	0.92 ± 0.30^{ab}	0.08 ± 0.08^a	0.00 ± 0.00^a
Col-0	**0.53 ± 0.13^a**	**0.37 ± 0.08^a**	0.33 ± 0.08^a	**0.32 ± 0.07^a**	0.81 ± 0.25^a	2.61 ± 0.13^b	0.88 ± 0.17^a	0.08 ± 0.04^a	0.56 ± 0.18^a	0.42 ± 0.25^a	0.63 ± 0.17^a	0.06 ± 0.06^a
Kas-0	3.63 ± 0.08^c	3.2 ± 0.15^c	—	2.77 ± 0.18^c	—	—	—	—	—	—	—	—
rks1-1	1.80 ± 0.16^b	0.40 ± 0.08^a	1.67 ± 0.26^b	0.63 ± 0.12^{ab}	2.25 ± 0.27^b	1.42 ± 0.28^a	2.25 ± 0.27^b	0.57 ± 0.13^b	2.67 ± 0.25^b	1.58 ± 0.30^b	1.25 ± 0.29^b	0.92 ± 0.43^a

*Numbers are the averages of the inoculation scores of three to six plants, and four leaves per plant, in two to three independent experiments. †Numbers are the averages of the inoculation scores of three to four plants, and four leaves per plant, in one experiment.—, not determined.For each *Xc* strain, different letters indicate different groups after pairwise comparisons using a Tukey honestly significant difference (HSD) test.

The dual resistance gene system *RRS1*/*RPS4* confers resistance to *XccCFBP6943* (race 6)

In response to the strain *XccCFBP6943*, a candidate gene was also identified on the basis of the GWA mapping results: *At5g45250* corresponding to SNP-5-18325565 being the most associated SNP. This gene encodes for RPS4 (RESISTANCE TO PSEUDOMONAS SYRINGAE4) (Gassmann *et al*., 1999; Hinsch and Staskawicz, 1996), a well-characterized R protein that confers recognition to the AvrRps4 effector from leaf-infecting *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst-avrRps4*) (Hinsch and Staskawicz, 1996; Sohn *et al*., 2012).

Because the accession Col-0 is highly susceptible to *XccCFBP6943* and the accession Ws-0 is rather resistant, we used the *rps4-21* mutant line (Narusaka *et al*., 2009b), identified in the Ws-0 background, to test whether $RPS4^{Ws}$ was involved in resistance to this strain (Fig. 4a). Both Ws-0 and the *rps4-21* mutant were inoculated with the strain *XccCFBP6943* and the disease index was scored at four time points after inoculation. At 7 dpi, the mutant showed a high level of susceptibility relative to the resistant wild-type (Fig. 4a,b). By measuring *in planta* bacterial growth in leaves at 7 dpi, we found that the *rps4-21* mutant line displayed the same level of susceptibility as Col-0, confirming that resistance to *XccCFBP6943* involves RPS4.

RPS4 has been described previously to cooperate genetically and molecularly with RRS1 (RESISTANCE TO RALSTONIA SOLANACEARUM1) (Birker *et al*., 2009; Deslandes *et al*., 2002; Narusaka *et al*., 2009b; Williams *et al*., 2014) in resistance to different pathogens, including *Pst-AvrRps4* and the root-infecting *Ralstonia solanacearum* bacterium expressing the PopP2 effector (Deslandes *et al*., 2003). We therefore investigated whether $RRS1^{Ws}$ and $RPS4^{Ws}$ could also function cooperatively in response to *XccCFBP6943*. The wild-type accession Ws-0, single *rps4-21* and *rrs1-1* mutants, and the *rps4-21 rrs1-1* double-mutant (Narusaka *et al*., 2009b) were inoculated with *XccCFBP6943* and *in planta* bacterial growth was measured in leaves at 7 dpi (Fig. 4a–c). Both the *rrs1-1* single mutant and *rps4-21 rrs1-1* double mutant displayed a similar level of susceptibility to the *rps4-21* single mutant. Therefore, the NLR (nucleotide-binding, leucine-rich repeat receptors) pair, $RPS4^{Ws}$ with $RRS1^{Ws}$, also operates against *XccCFBP6943*.

Finally, EDS1 (ENHANCED DISEASE SUSCEPTIBILITY1), a central regulator of basal resistance and of ETI mediated by resistance proteins belonging to the Toll-interleukin-1 receptor-nucleotide binding-leucine-rich repeat (TIR-NB-LRR) class of resistance proteins, forms protein complexes with RPS4 (Bhattacharjee *et al*., 2011; Heidrich *et al*., 2011). Thus, we tested whether EDS1 could also play a role in RPS4/RRS1-mediated resistance to *XccCFBP6943*. The *eds1-1* mutant exhibits a similar level of susceptibility to *rps4-21* and *rrs1-1* single and double mutants (Fig. 4), indicating that EDS1 is also required for RPS4/RRS1-mediated resistance to *XccCFBP6943*.

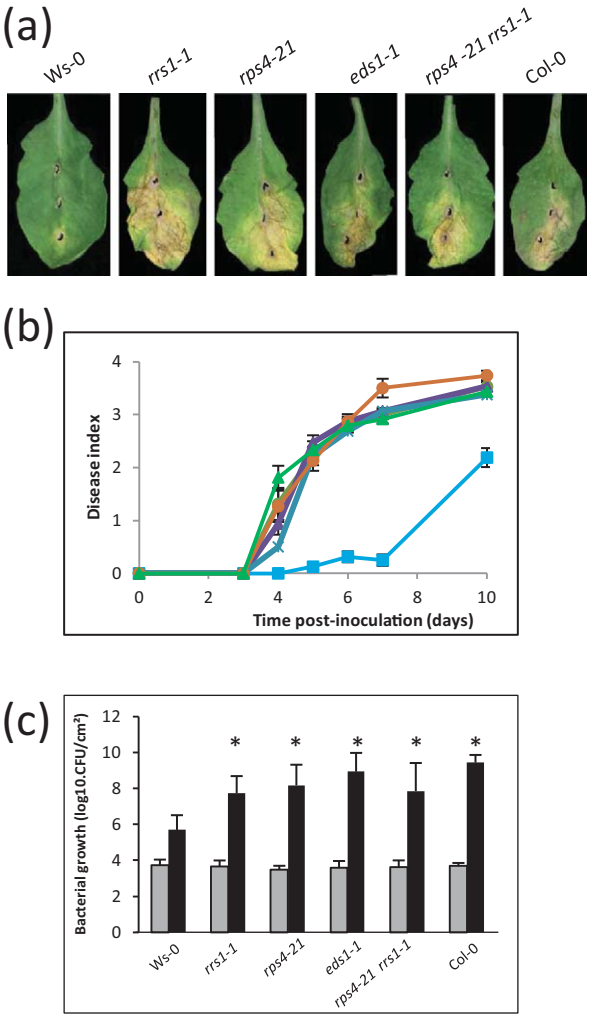

Fig. 4 The mutants *rrs1-1*, *rps4-21*, *rps4-21 rrs1-1* and *eds1-1* are susceptible to *XccCFBP6943*. (a) Disease symptoms were observed at 7 days post-inoculation (dpi) on leaves of wild-type plants (Ws-0 and Col-0) and mutants inoculated with the *XccCFBP6943* strain (race 6 defined by Fargier *et al*., 2011 and Vicente *et al*., 2001). (b) Time course evaluation of the disease index was performed in the mutants *rrs1-1* (blue cross), *rps4-21* (green circle), *eds1-1* (orange circle), *rps4-21 rrs1-1* double mutant (*rps4 rrs1*, purple cross), the parental line Ws-0 (blue square) and Col-0 (green triangle) after inoculation with *XccCFBP6943*. Means and standard errors were calculated from 10 plants (two independent experiments). (c) Bacterial growth measurement [colony-forming units (CFU)/cm^2 expressed on a $\log_{10}$ scale] in leaves of the mutants *rrs1-1*, *rsp4-21*, *rps4-21 rrs-1* or *eds1-1* and the parental line Ws-0. The susceptible accession Col-0 was included as a positive control. Bacterial growth was measured 0 dpi (grey bars) and 7 dpi (black bars) with *XccCFBP6943*. Data were collected from four independent experiments; each time point corresponds to six independent measurements, each on three to five individual plants (four leaves/plant). *Statistically significant difference using Kruskal–Wallis test ($P < 0.05$).

These results were confirmed using other strains of race 6 of *Xcc* (data not shown). However, RRS1, RPS4 and EDS1 seem to confer resistance specifically to this race, as the mutant lines are not significantly more susceptible than Ws-0 in response to other *Xcc* races in our experimental conditions (Table S3, see Supporting Information).

DISCUSSION

Although QDR is a durable and broad-spectrum form of resistance in plants, the identification of the genes underlying QDR and an understanding of the associated molecular mechanisms are still in their early stages (Balint-Kurti and Holland, 2015; Roux *et al*., 2014a). Recently, we have identified *RKS1* as a QDR gene conferring resistance in *A. thaliana* to the bacterial pathogen *Xc*. As for the majority of the few other QDR genes already identified, *RKS1* does not encode an NB-LRR resistance protein. Instead, *RKS1* encodes an atypical kinase (Huard-Chauveau *et al*., 2013; Roux *et al*., 2014b) that confers broad-spectrum resistance to most, but not all, *Xc* races.

Here, the power of nested GWA mapping to finely map genomic regions associated with the natural variation of quantitative traits was once again demonstrated in *A. thaliana*. We identified two new QTLs explaining between 30% and 51% of the phenotypic variation at the species level and conferring resistance to different races of *Xcc*: (i) the gene *At5g22540* conferring QDR to the strain *Xcc12824* (race 2); and (ii) the dual resistance gene system *RRS1/RPS4* conferring resistance to the strain *XccCFBP6943* (race 6). In the latter case, the *RRS1* and *RPS4* receptor gene pair that cooperates genetically and molecularly has been studied extensively (Birker *et al*., 2009; Narusaka *et al*., 2009b; Williams *et al*., 2014). RPS4 and RRS1 function as a heteromeric 'sensor–signalling' pair in ETI against AvrRps4 and PopP2, two unrelated effectors from the bacterial pathogens *Pseudomonas syringae* and *Ralstonia solanacearum*, respectively (Le Roux *et al*., 2015; Sarris *et al*., 2015; Williams *et al*., 2014). In addition, Narusaka *et al*. (2009b) have demonstrated that the *A. thaliana RCH2* (for recognition of *Colletotrichum higginsianum*) locus conferring resistance to *C. higginsianum* involves both RRS1 and RPS4. We show here that these two *R* genes located in a conserved head-to-head orientation confer QDR to the bacterial vascular pathogen *Xcc*. These results indicate that these *R* genes not only confer resistance to multiple pathogens with different infectious strategies, but are also involved in different forms of resistance, including QDR (as demonstrated previously for the *R* gene *Pi35* conferring resistance in rice to the fungal pathogen *Magnaporthe oryzae*; Fukuoka *et al*., 2014). Indeed, both *rrs1-1* and *rps4-21* null mutants display a quantitatively increased susceptibility to *XccCFBP6943* (disease index of 2.5–2.7; Table S3), whereas the most susceptible accessions display a disease index of about 4.0 (Fig. 2).

The susceptibility level of *rrs1-1 rps4-21* is similar to that of single mutants, suggesting that, as for the other pathogen species, RRS1 and RPS4 function cooperatively, possibly through effector sensing by RRS1, followed by the activation of RPS4 for resistance signalling (Cui *et al*., 2015; Griebel *et al*., 2014). The identification of the effector(s) from *Xcc* recognized by this (these) receptor(s) would be of primary interest in order to investigate the molecular mechanisms underlying this form of resistance. Interestingly, EDS1 is also required for RPS4/RRS1-mediated resistance to *XccCFBP6943*. Numerous NB-LRRs, including RPS4, interact in nuclear complexes with EDS1, which is considered to be a key resistance signalling component. Thus, EDS1 represents a molecular bridge connecting R protein effector activation to downstream defence reprogramming (Bhattacharjee *et al*., 2011; Heidrich *et al*., 2011; Kim *et al*., 2012), although direct interaction between AvrRps4 and EDS1 has not always been detected (Sohn *et al*., 2012). Although the QDR genes identified to date do not generally correspond to typical immune receptors and encode a broad range of molecular functions (Huard-Chauveau *et al*., 2013), in this case, as in a few other studies (Broglie *et al*., 2011; Fukuoka *et al*., 2014; Staal *et al*., 2006), the same perception and signalling components as ETI are utilized to confer a quantitative resistance to *Xcc*. In this case, the RRS1/RPS4-dependent ETI response can be considered as one of the multiple signalling pathways initiated by potential multiple recognition events accounting finally for QDR (Roux *et al*., 2014a).

Similarly, for *At5g22540*, the *mut540* mutant exhibits a quantitatively higher susceptibility to *Xcc12824* (disease index of 2.0) than the wild-type Col-0 accession, whereas the most susceptible accessions display a disease index of about 3.2 (Table 1). However, in this case, *At5g22540* gene expression (as evaluated using a primer located downstream of the T-DNA insertion) is highly enhanced in the mutant, suggesting that either: (i) the overexpression of the 3' end of the transcript affects the expression of *At5g22540*; or (ii) *At5g22540* encodes a quantitative disease susceptibility factor. In agreement with the latter, *At5g22540* expression is down-regulated during the infection process in the wild-type accession Col-0, suggesting that *At5g22540* might play an active role by suppression of resistance. It will be interesting to generate silenced and overexpressing *At5g22540* lines in order to confirm this hypothesis. In addition, in contrast with the well-characterized dual resistance gene system *RRS1*/*RPS4*, the gene *At5g22540* encodes a protein with an unknown function. Together with *RKS1*, which encodes an atypical kinase, this study highlights the diversity of molecular functions underlying QDR to *Xc*. Such a complexity of molecular functions for the genetic control of QTL-mediated resistance to the same pathogen has also been demonstrated in wheat to stripe rust (Fu *et al*., 2009; Krattinger *et al*., 2009), in rice to *M. oryzae* (Fukuoka *et al*., 2015) and in soybean to nematode (Cook *et al*., 2012; Liu *et al*., 2012).

QDR genes typically provide broad-spectrum resistance. For example, wheat *Yr36* provides high-temperature-dependent QDR to eight stripe rust races (Fu *et al*., 2009) and Lr34 provides resistance to two rust diseases of wheat, *Puccinia striiformis* and *Puccinia triticina* (Krattinger *et al*., 2009). Broad-spectrum resistance conferred by QDR genes may relate to the perception and response pathways to well-conserved microbial signatures, such as conserved effectors and PAMPs. For instance, expression of the PRR-encoding gene *EFR* confers QDR to adapted and non-adapted pathogens in several plant families (Lacombe *et al*., 2010). Alternatively, QDR genes may correspond to downstream effector response components: this is the case for most QDR genes identified to date. These could either act at the intersection of several effector perception pathways or represent common host targets indirectly manipulated by different effectors (Roux *et al*., 2014a). In the case of QDR to *Xcc*, we identified several genes displaying strikingly different ranges of specificity (Fig. 5): (i) *RKS1* confers resistance to races 1, 3, 5, 7 and 9 (and, to a much lesser extent, race 8) and to strains belonging to *Xcr*, but not to races 2 and 4; (ii) *At5g22540* is involved in resistance to different strains of *Xcc* belonging to races 1, 2, 3 and 4; and (iii) the *RRS1/RPS4* gene pair confers resistance only to race 6 of *Xcc*. However, all only confer partial resistance to *Xc* (Tables 1 and S3). Interestingly, both *RKS1* and *At5g22540* confer resistance to races 1 and 3, suggesting that they cooperate through dependent or independent pathways to mount resistance against these races. To our knowledge, this study provides the first example of the identification of several QDR genes conferring, together, resistance to all races of a pathogen.

Intensive genetic mapping of resistance loci operating for pathogens for which QDR is the predominant form of plant resistance has highlighted, in a large number of cases, a complex genetic architecture underlying the control of QDR (Cook *et al*., 2012; Fukuoka *et al*., 2015; Roux *et al*., 2014a). However, because of their small phenotypic effects, the identification of the genes underlying QTLs remains a challenge. In addition, as for *RKS1* (Huard-Chauveau *et al*., 2013), our approach of nested GWA mapping suggests an allelic series for both QTLs identified in this study, reinforcing the importance of exploring the complete allelic diversity at a QDR gene to fully understand the genetic control of QTL-mediated resistance (Fukuoka *et al*., 2014). Finally, as observed previously for *RKS1* (Huard-Chauveau *et al*., 2013), numerous populations across the native range of *A. thaliana* are polymorphic for the two major QTLs identified in this study (Fig. S2, see Supporting Information), stressing the need to study the adaptive dynamics of multiple QTL-mediated resistance to *Xc* at a small spatial scale. Linking the dissection of QDR pathways to eco-evolutionary dynamics at a local scale is of major importance for the development of realistic models aimed at the evaluation of the suitability of the pyramiding of QDR genes for crop disease management in an agro-ecological context.

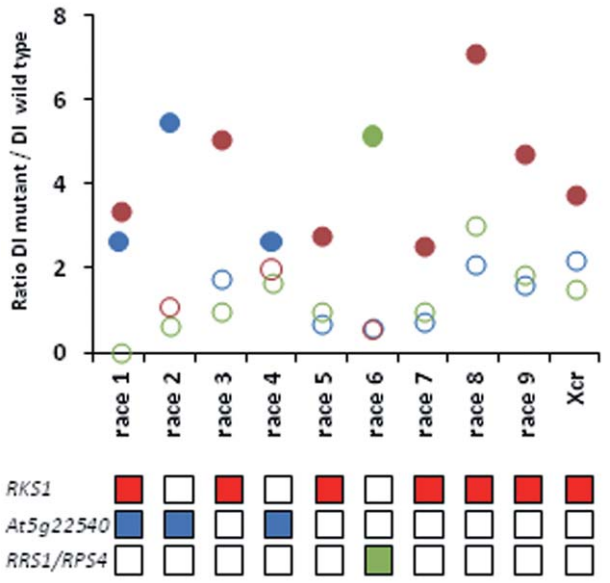

Fig. 5 Quantitative resistance conferred by the genes *RKS1*, *At5g22540* and *RPS4/RRS1* to the nine races of *Xanthomonas campestris* pv. *campestris* (*Xcc*) (as defined by Fargier *et al*., 2011 and Vicente *et al*., 2001), as well as to *Xanthomonas campestris* pv. *raphani* (*Xcr*). Top: ratio of disease index (DI) at 7 days post-inoculation of mutant against disease index (DI) of corresponding wild-type. Data correspond to one to three independent experiments, each on three to five individual plants (four leaves/plant). For each *X. campestris* strain, the ratios of *rks1-1*/Col-0, *mut540*/Col-0 and *rps4-21 rrs1-1*/Ws-0 are represented by red, blue and green circles, respectively. Filled circles indicate significant differences between a mutant line and its corresponding wild-type after pairwise comparisons using a Tukey honestly significant difference (HSD) test (see Tables 1 and S3). The strains correspond to *Xcc1869* (race 1), *Xcc12824* (race 2), *Xcc568* (race 3), *Xcc147* (race 4), *Xcc1712* (race 5), *Xcc6943* (race 6), *Xcc4953* (race 7), *Xcc1124* (race 8), *Xcc8004* (race 9) and *Xcr756C* (Xcr). Bottom: summary of broad-spectrum resistance conferred by the genes *RKS1*, *At5g22540* and *RPS4/RRS1*. Filled squares indicate significant differences between a mutant line and its corresponding wild-type after pairwise comparisons using a Tukey HSD test (see Tables 1 and S3).

EXPERIMENTAL PROCEDURES

Bacterial material

The strains *Xcc12824* and *XccCFBP6943* belong to races 2 and 6, respectively, as defined by Fargier and Manceau (2007), Fargier *et al*. (2011) and Vicente *et al*. (2001). Spontaneous resistant clones of *Xcc12824* and *XccCFBP6943* were selected on Kado medium (Kado and Heskett, 1970) supplemented with 50 mg/mL rifampicin.

Broad-spectrum resistance was estimated by inoculation tests with different races of *Xcc* and *Xcr* [CIRM-CFBP collection, INRA Angers, France]. All *Xcc* and *Xcr* strains were grown on Kado medium. Cultures of *Xcc568* (LUX) were supplemented with 50 mg/mL rifampicin and 25 mg/mL kanamycin.

Plant material

To investigate the natural variation of resistance to *Xcc*, we used a set of *A. thaliana* natural accessions. For *Xcc12824*, we used the same set of 384 natural accessions that have been phenotyped previously for QDR to strain *Xcc568* (race 3; Huard-Chauveau *et al*., 2013). This set of 384 natural accessions includes 179 worldwide accessions (WA), 188 French accessions (FA), as part of the French RegMap (Bergelson and Roux, 2010), and 17 accessions that are both WA and FA. For *XccCFBP6943*, we used a subset of 176 WA. All the 384 natural accessions were genotyped for 214 051 SNPs evenly spaced across the genome (Horton *et al*., 2012).

Some mutants used in this study were identified in the SALK library (http://signal.salk.edu) and are in the *A. thaliana* accession Columbia (Col-0) background: the lines *N654537*, *N664670* and *N667597* for gene *At5g22500*, line *N670195* for gene *At5g22510*, lines *N682427* and *N681954* for gene *At5g22530* and line *N667062* (*mut540* mutant) for gene *At5g22540*. The position of the T-DNA insertion was confirmed by polymerase chain reaction (PCR) using T-DNA-LB (5'CCCTTTAGGGTTCCG ATTTAGTGCT) and 540-seq848F (5'ATAGTAATGGTGTGCTTCAC) primers and sequencing. The *rrs1-1*, *rps4-21*, *rrs1-1 rps4-21* (Narusaka *et al*., 2009b) and *eds1-1* (Parker *et al*., 1996) mutants originated from the accession Wassilewskija (Ws-0).

Plant inoculation and phenotyping

Plants were grown on Jiffy pots under controlled conditions (Lacomme and Roby, 1996). The virulence of *Xcc* strains and other pathovars was tested on 28-day-old plants after inoculation by piercing and scoring of the symptoms according to a scale from 0 to 4, as described in Meyer *et al*. (2005).

In planta bacterial growth analysis [colony forming units (CFU)/cm^2, expressed on a $\log_{10}$ scale] was performed as described by Froidure *et al*. (2010). Because *Xcc* is a vascular bacterial species, bacterial growth was measured at 0 and 7 dpi by piercing inoculation with either *Xcc12824* or *Xcc6943* (rifampicin-resistant derived clones) at a distance from the inoculation zone (at the tip of the inoculated leaves). Data were collected from at least two independent experiments; each time point corresponds to six independent measurements, each on three to five individual plants (four leaves per plant). At the inoculation site (base of the inoculated leaves), bacterial growth was measured and found to be similar in the different lines (*Xcc12824*: 5.3 ± 0.2 to 5.4 ± 0.2 $\log_{10}$ CFU/cm^2 at T0; 9.4 ± 1 to 10.1 ± 1 $\log_{10}$ CFU/cm^2 at 7 dpi; *XccCFBP6943*: 5.5 ± 0.1 to 5.8 ± 0.3 $\log_{10}$ CFU/cm^2 at T0).

Natural variation of QDR

Experimental design

For *Xcc12824*, an experiment with 1728 plants was set up at the University of Lille 1 (France) according to a completely randomized design involving four experimental blocks, each block being an independent randomization of one replicate per accession. Infected plants were placed in plastic mini-glasshouses, including two control accessions, Col-5 (resistant) and Kas-1 (susceptible), in the same positions within each mini-glasshouse. Mini-glasshouses were placed in phytotrons (22 °C, 9-h photoperiod, 100% humidity).

For *XccCFBP6943*, an experiment with 768 plants was set up at INRA in Castanet-Tolosan (France) according to a completely randomized design involving four experimental blocks, each block being an independent randomization of one replicate per accession. Infected plants were placed in plastic trays, including two control accessions, Col-5 (resistant) and Kas-1 (susceptible), in the same positions within each tray. Trays were placed in growth chambers (22 °C, 9-h photoperiod, 100% humidity).

Statistical analyses

For each *Xcc* strain, we used the following general linear model (GLM procedure in SAS9.1; SAS Institute Inc., Cary, NC, USA) to explore the natural genetic variation of the disease index:

$$\text{disease index}_{ij} = \mu + \text{block}_i + \text{accession}_j + \text{cov}_{Col\text{-}5} + \text{cov}_{Kas\text{-}1} + \varepsilon_{ij} \quad (1)$$

where μ is the overall mean of the phenotypic data, 'block' accounts for differences among the four experimental blocks, 'accession' corresponds to the differences among the natural accessions, $\text{cov}_{Col\text{-}5}$ and $\text{cov}_{Kas\text{-}1}$ are covariates accounting for mini-glasshouse (or plastic trays) effects and ε is the residual term. Normality of the residuals was not improved by transformation of the data. The least-square mean (LSmean) was obtained for each natural accession and was subsequently used for GWA mapping analyses.

Broad-sense heritabilities (H^2) were estimated from the mean square (MS) of Equation (1) using a formula adapted from Gallais (1990).

GWA mapping

In order to fine map the genomic regions associated with natural disease index variation, we ran a mixed model implemented in the software EMMAX (Efficient Mixed-Model Association eXpedited; Kang *et al*., 2010). This mixed model includes a genetic kinship matrix based on the 214 051 SNPs as a covariate to control for population structure in the mapping panel.

RNA isolation and quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR)

RNA extraction and Q-RT-PCR analysis were performed as described by Froidure *et al*. (2010) using leaves from healthy plants. Q-RT-PCR analysis of transcript accumulation in wild-type Col-0 and *mut540* mutant Arabidopsis leaves was performed using two pairs of primers: QF4-540 (TCTTCTGTTTGCGGCTTTG) + 3'UTR-R (CAAGAATCAAAACACTTAACTT) and QF4-540 (TCTTCTGTTTGCGGCTTTG) + QR4-540 (AGTCTTTTCTTTTGG AGGACG) (Fig. S1).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Molecular characterization of the T-DNA mutant line *mut540*. (a) Schematic representation of the insertion site of the T-DNA in the mutant line *mut540* (SALK-113262C). The T-DNA insertion occurs at position 1253, as shown by sequencing of the T-DNA borders and of the flanking regions. The location of the primers used for gene expression analysis is represented. (b) *At5g22540* gene expression analysis in leaves of the wild-type accession (Col-0) and the mutant line (*mut540*) by gel analysis of polymerase chain reaction (PCR) products using the primers [QF4-540 + 3'UTR-R]. (c) Time course analysis of *At5g22540* expression by quantitative reverse transcription (RT)-PCR (using the primers QF4-540 + QR4-540) in the wild-type (black diamonds) relative to the *mut540* line (grey triangles) after inoculation with *Xcc12824*. Means and standard errors were calculated from nine leaves in one representative experiment.

Fig. S2 Geographical distribution of polymorphisms associated with quantitative disease resistance to either *Xcc12824* or *Xcc6943*. (a) Geographical distribution of the two alleles at the top SNP 5_7481857 detected by genome-wide association (GWA) mapping for quantitative disease resistance to *Xcc12824*. Alleles 'A' and 'C' are associated with resistance and susceptibility to *Xcc12824*, respectively. (b) Geographical distribution of the two alleles at the top SNP 5_18325565 detected by GWA mapping for quantitative disease resistance to *XccCFBP6943*. Alleles 'G' and 'A' are associated with resistance and susceptibility to *XccCFBP6943*, respectively. Maps were based on 948 natural accessions with accurate GPS coordinates, genotyped for 214 051 single-nucleotide polymorphisms (SNPs) (Hancock *et al.*, 2011) and generated with the *R* packages 'maptools' and 'plotrix'. The size of the circles depends on the number of accessions genotyped for 214 051 SNPs in the collection sites.

Table S1 Mutants identified in the *At5g22540* locus and their phenotype in response to inoculation with *Xcc12824*, at 7 days post-inoculation.

Table S2 Disease index (10 days post-inoculation) of the *mut540* mutant after inoculation with different strains belonging to *Xanthomonas campestris* pv. *campestris* (*Xcc*) races (as defined by Fargier *et al.*, 2011 and Vicente *et al.*, 2001) and with the pathovar *raphani* (*Xcr*).

Table S3 Disease index of the *rps4-21*, *rrs1-1*, *eds1-1*, *rks1-1* single mutants and the *rps4-21 rrs1-1* double mutant after inoculation with different strains belonging to *Xanthomonas campestris* pv. *campestris* (*Xcc*) races (as defined by Fargier *et al.*, 2011 and Vicente *et al.*, 2001) and with the pathovar *raphani* (*Xcr*). Disease index at 7 days post-inoculation.